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(54) [Title of the Invention] Interferon-gamma Production Promoter

(57) [Summary]

[Object] To provide a novel drug that is useful in treating or preventing diseases in which it is desirable to promote the production of interferon-gamma.

[Constitution] An interferon-gamma production promoter whose active ingredient is a peptide having the amino acid sequence Thr-Asp-Asp-Thr-Ala-Ile-Val-Leu-Leu-Lys.

## **[Claims]**

**[Claim 1]** An interferon-gamma production promoter whose active ingredient is a peptide having the amino acid sequence (Sequence No. 1) Thr-Asp-Asp-Thr-Ala-Ile-Val-Leu-Leu-Lys.

**[Claim 2]** The interferon-gamma production promoter according to Claim 1, which promotes the production of interferon-gamma in normal liver cells.

**[Claim 3]** An antiviral agent based on promoting the production of interferon-gamma.

## **[Detailed Description of the Invention]**

### **[0001]**

**[Technological Field of the Invention]** The present invention relates to an interferon-gamma production promoter whose active ingredient is a peptide expressed by Sequence No. 1 on the sequence chart.

### **[0002]**

**[Prior Art and Problems That the Invention Is Intended to Solve]** The living organism combats infection by bacteria, viruses, and the like through an immune response. The humoral factors that produce the macrophages and lymphocytes, which serve as the bearers of this immune response, are generally termed cytokines.

**[0003]** There is a group of substances called interleukins (referred to hereinafter as "IL") among the substances classified as cytokines. These are currently classified as interleukins 1-15 (referred to respectively hereinafter as "IL-1" to "IL-15").

**[0004]** IL-1 is a substance that used to be called lymphocyte-activating factor. It exhibits various bioactivities in trace quantities of  $10^{-10}$  mol or less, and is known to be intimately involved in the mechanisms of homeostasis in the body. Attention turned to the bioactivities of IL-1, and research has advanced on the utilization of IL-1 for purposes such as pharmaceuticals. However, since IL-1 has a variety of bioactivities, effects other than the target effect appeared simultaneously when it was administered to the body in high doses, and there was a risk of disrupting the body's homeostasis. Research consequently advanced on the utilization of substances that have only some of the bioactivities of IL-1 (generally termed "IL-1-like substances") as substitutes for IL-1.

[0005] A polypeptide (referred to hereinafter as "P-10") expressed by Sequence No. 1 was discovered as an IL-1-like substance. It was confirmed to be biologically active in the sense that it enhances nonspecific antibody production and potentiates thymus cells in the joint presence of concavalin A (JP (Kokai) 3-170498).

[0006] Furthermore, P-10 has been reported to promote the growth of normal liver cells but to suppress the growth of liver cancer cells (*Cytokine*, Vol. 6, No. 3, 1994: pp. 265-271 (The substance called P-10.2 in this reference is the same substance as P-10 in the present specification)). P-10 was discovered to have a new bioactivity different from that of an IL-1-like substance because it is stated in the above reference that IL-1 does not suppress the growth of liver cancer cells.

[0007] Therefore, the object of the present invention is to explore unknown bioactivities of P-10, to clarify the effects of P-10 on liver cells that are not shared by immunocompetent cells such as lymphocytes, and to discover new uses for P-10.

[0008]

**[Means Used to Solve the Above-Mentioned Problems]** As a result of research conducted in order to attain the above-stated goals, the inventors perfected the present invention upon discovering that P-10 specifically promotes the production of interferon-gamma (referred to hereinafter as IFN- $\gamma$ ).

[0009] Specifically, the first essential feature of the present invention resides in an IFN- $\gamma$  production promoter whose active ingredient is a peptide (specifically, P-10) having the amino acid sequence (Sequence No. 1) Thr-Asp-Asp-Thr-Ala-Ile-Val-Leu-Leu-Lys. The second essential feature of the present invention resides in an antiviral agent based on the effect whereby P-10 promotes production of IFN- $\gamma$ .

[0010] The present invention will be explained in detail below. The IFN- $\gamma$  production promoter of the present invention acts on the cells of the living organism and causes IFN- $\gamma$  that has the bioactivities discussed hereinbelow to be produced in the cells.

[0011] IFN is a substance produced by animal cells that have been stimulated by viruses, nucleic acids, and the like, and is an antiviral protein secreted outside the cells. Subsequent research clarified that it has not only an antiviral effect, but also a variety of activities such as an

antitumor effect and the ability to activate the immune system. IFN is broadly classified as  $\alpha$ -,  $\beta$ -, and  $\gamma$ -types. The type produced by T-cells and the like due to stimulation by substances such as lectins and mitogens in addition to viruses is called  $\gamma$ -type (IFN- $\gamma$ ). IFN- $\gamma$  exhibits an antiviral effect, but has no structural similarity to IFN- $\alpha$  or - $\beta$ . It also differs in the types of receptors on the cells on which it acts, and its properties and effects are considerably different from those of IFN- $\alpha$  and - $\beta$ . Consequently, IFN- $\gamma$  is currently ranked as an interleukin even though it has an antiviral effect.

[0012] The following bioactivities of IFN- $\gamma$  are thus known: the antiviral effect mentioned above, an antitumor effect, an effect that promotes induction of cytotoxic T-lymphocytes, an effect that promotes natural killer cell activity, a neutrophil-activating effect, a macrophage-activating effect, an effect that promotes MHC class II expression, an effect that promotes IL-2 receptor expression, an effect that promotes Fc receptor expression, and the like.

[0013] IFN is also by nature a biological substance that serves as a first line of defense of the body against foreign matter. It has high, very selective toxicity and is extremely safe for the body.

[0014] A drug (referred to hereinafter as "the drug of the present invention") whose active ingredient is the P-10 of the present invention, which is an ingredient that promotes the secretion of IFN- $\gamma$  by stimulating living cells, can naturally elicit the bioactivity possessed by IFN- $\gamma$ . Therefore, the drug of the present invention can be used as an antiviral agent, an antitumor agent, or a drug designed to combat bacterial infections.

[0015] The safety of P-10 as an active ingredient of the drug of the present invention was confirmed by the above-cited reference (Cytokine) and by Test Case 1, which is described below. The effect of promoting production of IFN- $\gamma$  was confirmed by the experiments of Test Cases 2 and 3.

[0016] The drug of the present invention can be used to treat or prevent diseases of humans and other warm-blooded animals in which it is desirable to promote the production of IFN- $\gamma$ , e.g., cancer, viral infections, bacterial infections (such as liver abscess and hepatic amoebiasis), and the like. It can be used to a particular advantage in the treatment of cancer and the treatment or prevention of viral infections.

[0017] The drug of the present invention can be used in various forms, e.g., oral preparations such as tablets, granules, capsules, and fine granules, and percutaneous preparations such as injections, plasters, and ointments, as well as permucosal preparations, implants, and ophthalmic solutions.

[0018] Pharmaceutically permissible excipients, auxiliaries, and other active drug ingredients can be added in making the drug of the present invention into a preparation. Examples of suitable excipients include sodium chloride, glycine, lactose, mannitol, sorbitol, sucrose, starch, dextran, and gelatin.

[0019] The P-10 content of the drug of the present invention varies depending on the drug form. However, it is generally preferred that it be 5-100% by weight in relation to the weight of the preparation in the case of solid and semi-solid preparations, and 0.01-10% by weight in the case of liquid preparations.

[0020] The dose of the drug of the present invention can be varied over a wide range depending on the type of targeted warm-blooded animal (including humans), type of condition to be treated or prevented, severity of the symptoms, administration site, drug form, judgment of the physician, and the like. Although the upper and lower limits of the dose are not fixed, the amount of P-10 as an active ingredient of the drug of the present invention is generally 5 µg-10mg/kg/day, and preferably 10 µg-4 mg/kg/day. Furthermore, the drug of the present invention can be administered once a day or several times a day in such a way as to reach the aforementioned dose per day.

[0021] The drug of the present invention can be used in combination with other drugs, e.g., therapeutic agents such as cancer suppressants, antiviral agents, antibiotics, and chemotherapeutics, and nutritional supplements such as vitamins and sugar solutions, as long as these do not interfere with the pharmacological effects of P-10 as an active ingredient.

[0022] The antiviral agent (referred to hereinafter as "the antiviral agent of the present invention") based on the effect whereby production of IFN-γ is promoted by P-10 as the second essential feature of the present invention is the same as the drug of the present invention described above except that the condition to be treated or prevented is a viral infection. Specifically, its dosage forms, P-10 content, doses, and the like are as explained above for the drug of the present invention.

[0023] Examples of viral infections in which the antiviral agent of the present invention can be used include viral hepatitis caused by the hepatitis B virus, hepatitis C virus, or hepatitis D virus, but are not limited to these.

[0024]

[Working Examples] The present invention is explained in greater detail below through working examples.

[0025] Test Case 1: Effects of P-10 on the growth of normal mouse liver cells

A test was conducted to determine how P-10 affects the cell growth of NCTC Clone 1469 (referred to hereinafter as "Clone 1469"), which is a normal mouse liver cell line. The cell growth response was measured by the uptake of bromodeoxyuridine.

[0026] Cell growth was measured using an immunohistochemical detection system (Cell Proliferation Detection Kit, made by Amersham). P-10 was added to Clone 1469 cells ( $2 \times 10^4$  cells/mL). The cells were cultured in 24-well flat-bottomed plates (made by Corning). A quantity of 1  $\mu$ g/mL of bromodeoxyuridine was added and the cells were cultured for six hours. After being washed three times with phosphate-buffered physiological saline (PBS, pH 7.2), the cells were immobilized for 30 minutes by acid-ethanol. Then, 100  $\mu$ L/well of anti-bromodeoxyuridine monoclonal antibody and 100  $\mu$ L/well of peroxidase-labeled anti-mouse IgG were added. The cells that had taken up bromodeoxyuridine were stained by adding DAB (diaminobenzidine) that contained 0.03%  $H_2O_2$  and 0.03%  $NiCl_2$ , and measured under the microscope.

[0027] The test results are shown in Table 1 below.

[0028]

[Table 1]

Table 1. Effects of P-10 on the cell growth of Clone 1469

P-10 concentration ( $\mu$ g/mL)	Bromodeoxyuridine uptake by Clone 1469 (%)
0	45.5 $\pm$ 2.8
10	73.8 $\pm$ 4.0*

\*:  $p < 0.001$

**[0029]** Based on the results in Table 1, it can be seen that P-10 promotes the growth of normal liver cells.

**[0030]** Test Case 2: Effects of P-10 on the cytokine secretion of liver cancer cells and normal liver cells

In order to investigate how P-10 affects the secretion of cytokines known to be secreted by liver cells, variations in the number of cytokine-secreting cells of Clone 1469 and MH134, which is a mouse liver cancer cell line, were measured by ELISPOT (enzyme-lined immunospot).

**[0031]** Specifically, IFN- $\gamma$  derived from helper T-cells 1 ( $T_h1$ ), as well as IL-4, IL-5, and IL-10 derived from helper T-cells 2 ( $T_h2$ ), were detected as the cytokines.

**[0032]** P-10 was added to the Clone 1469 cells and MH134 cells, and the cells were cultured for 42 hours. After the culture broth had been centrifuged, the cells were collected and the respective cells were suspended in a concentration of  $5 \times 10^4$  cells/0.5 mL in RPMI 1640 culture medium that contained 1% fetal calf serum (made by Nissui Pharmaceutical). The suspension was aliquotted into each well (capacity 0.5 mL) of a Millicell (12 mm in diameter) and cultured for six hours at 37°C in the presence of 5% carbon dioxide in 24-well plates (made by Corning). After six hours, the culture broth was discarded, and the adhering cells were removed by gently scraping the surface of the Millicell membrane with a cell scraper (made by Sumitomo Bakelite). The surface of the Millicell membrane was washed three times by Tris-buffered physiological saline (TBS, pH 7.6).

**[0033]** Next, TBS that contained 2% hydrogen peroxide was added to the Millicell membrane. After a 10-minute reaction, the Millicell membrane was washed five times with TBS that contained 1% Tween 20 (made by Sigma) (TBST). TBS that contained 3% bovine serum albumin (BSA) (made by Sigma) was added to the Millicell membrane, a reaction was conducted for one hour at 37°C, and the membrane in each well of the Millicell membrane was blocked. The Millicell membrane was then washed three times by TBST, and 0.2 mL of biotin-labeled anti-mouse IFN- $\gamma$  antibody, biotin-labeled anti-mouse IL-4 antibody, biotin-labeled anti-mouse IL-5 antibody, or biotin-labeled anti-mouse IL-10 antibody (all made by Pharmingen, 500 $\times$  dilutions) was added to each well. After standing overnight, the Millicell membrane was washed four times with TBST.

[0034] Avidin-peroxidase (made by Vector Laboratory, ABC Kit) was added to each well, a reaction was conducted for 30 minutes, and the wells were washed three times by TBS. After 0.2 mg/mL of diaminobenzidine (DAB) and TBS that contained 0.003% hydrogen peroxide were added and a reaction was conducted for 10 minutes, the spots that formed in each well were counted under the microscope.

[0035] The number of spots that formed corresponded to the number of cells that produced each of the cytokines.

[0036] The results of the test are shown in Table 2.

[0037]

[Table 2]

Table 2. Effects of P-10 on cytokine secretion in Clone 1469 and MH134

Cell type	P-10 concentration (μg/mL)	Number of cytokine-secreting cells (per 10 <sup>5</sup> cells)			
		IL-4	IL-5	IL-10	IFN-γ
Clone 1469	0	100±20	135±15	50±10	240±80*
	10	115±5	95±5	55±15	510±70*
MH134	0	150±10	780±160	170±50	260±100
	10	160±80	870±110	149±30	370±70

\*: p<0.001

[0038] It can be seen from the results in Table 2 that cells that secrete cytokines are present in Clone 1469 cells and MH134 cells even without stimulation by P-10.

[0039] Only the number of cells that secreted IFN-γ was significantly increased by the addition of P-10 in Clone 1469, which is a normal liver cell line. IL-4, IL-5, and IL-10 did not differ significantly from the control group. Addition of P-10 produced no significant variations in any of the cytokines in MH134, which is a liver cancer cell line.

[0040] It can be seen based on the above results that P-10 specifically promotes the production of IFN-γ in normal liver cells.



**[0041]** Test Case 3: Effects of P-10 on the IFN- $\gamma$  secretion of normal liver cells

The above Test Case 2 confirmed that addition of P-10 promoted IFN- $\gamma$  secretion in normal liver cells (Clone 1469). Therefore, the effects of the P-10 concentration on the IFN- $\gamma$  secretion of normal liver cells were investigated in this test.

**[0042]** The test was conducted in the same way as Test Case 2, except that the P-10 concentrations were as shown below in Table 3. The results of the test are shown in Table 3.

**[0043]**

**[Table 3]**

Table 3. Effect of the P-10 concentration on IFN- $\gamma$  secretion of Clone 1469

P-10 concentration ( $\mu\text{g/mL}$ )	Number of INF- $\gamma$ -secreting cells (per $10^5$ cells)
0	240 $\pm$ 80
1	200 $\pm$ 40
10	510 $\pm$ 70*
50	760 $\pm$ 60*
100	710 $\pm$ 110*

\*:  $p < 0.001$

**[0044]** It can be seen from the results in Table 3 that cells that secreted IFN- $\gamma$  were produced in amounts that were respectively 2.1 times, 3.2 times, and 3.0 times greater in comparison with the control group when 10, 50, or 100  $\mu\text{g/mL}$  of P-10 was added.

**[0045]** The results of the above test cases indicate that P-10 specifically promotes the production of IFN- $\gamma$  by stimulating normal liver cells.

**[0046]**

**[Merits of the Invention]** The drug of the present invention is highly safe for normal somatic cells and can promote the production of IFN- $\gamma$  in normal somatic cells, especially liver cells. It can be expected to have useful pharmacological effects based on the bioactivities of IFN- $\gamma$ .

**[0047]** Specifically, the present invention provides a novel drug that is useful in the treatment and prevention of diseases in which it is desirable to promote the production of IFN- $\gamma$ .

[0048]

[Table 4]

[Sequence Chart]

Sequence No. (SEQ ID NO): 1

Sequence length: 10

Sequence type: amino acid

Sequence

Thr Asp Asp Thr Ala Ile Val Leu Leu Lys

1

5

10